ISSN (print) 0093-4666 © 2013. Mycotaxon, Ltd. ISSN (online) 2154-8889

MYCOTAXON

http://dx.doi.org/10.5248/123.95

Volume 123, pp. 95–101 *January–March 2013*

Tuber subglobosum and *T. wenchuanense* — two new species with spino-reticulate ascospores

Li Fan[1*], Jin-Zhong Cao[2] & Cheng-Lin Hou[1]

[1] *College of Life Science, Capital Normal University, Xisanhuanbeilu 105, Haidian, Beijing 100048, China*

[2] *Institute of Mycology, Jilin Agricultural University, Changchun 130118, China*

* Correspondence to: *fanli@mail.cnu.edu.cn*

Abstract — Two new *Tuber* species from China with spino-reticulate ascospores are described and illustrated. *Tuber subglobosum* is characterized by its subglobose ascospores with distinct spino-reticulate ornamentation, and *T. wenchuanense* is recognized by the very irregular spino-reticulate ornamentation on the ascospore surface, with the spines connected to each other at the base and forming U-shaped ridges at the apex. ITS sequence analyses support both new species.

Key words — *Ascomycota*, *Tuberaceae*, taxonomy

Introduction

Arguably the most useful feature for distinguishing *Tuber* species is the type of ascospore ornamentation. Some European species such as *T. aestivum* Vittad. and *T. magnatum* Picco have a more or less regular reticulum (mesh) covering the spore surface (Riousset et al. 2001). The other species group, which includes *T. melanosporum* Vittad. and *T. rufum* Vittad., has ascospores covered by spines. In contrast, many Chinese species have spino-reticulate ornamentation on the ascospore surface where a regular arrangement of spines is linked by U-shaped ridges. This was first described by Liu (1985) for *T. taiyuanense* B. Liu and again by Wang (1990) for *T. liaotongense* Y. Wang from Northeast China. More recently, other species with spino-reticulate ascospore ornamentation that have been described include *T. huidongense* Y. Wang, *T. microspermum* L. Fan & J.Z. Cao, *T. microspiculatum* L. Fan & Yu Li, *T. polyspermum* L. Fan & C.L. Hou, *T. sinoalbidum* L. Fan & J.Z. Cao, and *T. umbilicatum* Juan Chen & P.G. Liu (Chen et al. 2005, Fan et al. 2012a,b; Wang & He 2002).

Curiously, so far only one European species, *Tuber malacodermum* E. Fischer, and two North American species, *T. spinoreticulatum* Uecker & Burds. and

T. lyonii Butters, have been described with spino-reticulate ascospores (Chen et al. 2005). In this paper two additional species of *Tuber* with spino-reticulate ascospores are described from China.

Materials & methods

Morphological studies

The specimens of one *Tuber* species (collected in Sichuan Province) were already dried and deposited in HMAS (Herbarium Mycologicum Academiae Sinicae, Institute of Microbiology, Chinese Academy of Sciences). The specimens of the other new species were collected fresh from Sichuan and Yunnan Provinces and deposited in BJTC (Herbarium Biology Department, Capital Normal University). Macroscopic characteristics are described from both fresh and rehydrated specimens. Microscopic characters were described from razor-blade sections mounted in 3% KOH, Melzer's reagent, or 0.1% (w/v) cotton blue in lactic acid. For scanning electron microscopy (SEM), spores were scraped from the dried gleba, placed onto double-sided tape, mounted directly on an SEM stub, coated with gold-palladium, and then examined and photographed with a HITACHI S-4800 SEM.

Molecular methods

Herbarium samples were crushed by shaking for 3 min at 30 Hz (Mixer Mill MM 301, Retsch, Haan, Germany) in a 1.5 ml tube together with one 3 mm diameter tungsten carbide ball. Total genomic DNA was then extracted using the PeqLab E.Z.N.A. Fungal DNA kit following the manufacturer's protocol. The ITS region was amplified with PCR using the primers ITS1/ITS4 (White et al. 1990). PCR was performed in 50 µl reactions containing DNA template 2 µl, primer (10 µM) 2 µl each, 2× Master Mix

Table 1. *Tuber* specimens and sequence numbers used in molecular studies.

Species	Voucher specimen	ITS	ITS1	ITS2
T. aestivum	C. TaeW028I-E157 (UPS)	AJ888118	–	–
	C. TaeW064S-W140 (UPS)	AJ888062	–	–
T. huidongense	Thui-pzh08	DQ486032	–	–
	Thui-pzh09	DQ486031	–	–
T. liaotongense	IFS Y. Wang 88059	–	DQ478672	DQ478634
	IFS Y. Wang 88061	–	DQ478671	DQ478635
T. lyonii	GB 112	EU394704	–	–
	MOHARCN5Cfb	EU268568	–	–
T. rufum Picco	Truf-eu01	DQ329375	–	–
	R1	AF106892	–	–
	Morphotype 5	AY940646	–	–
	1506	AY112894	–	–
T. spinoreticulatum	RH158	GQ221454	–	–
	U188	FJ809884	–	–
T. subglobosum	BJTC FAN153	JX267043	–	–
T. taiyuanense	T42_HMAS 75888	GU979033	–	–
	HMAS 60234	–	DQ478664	DQ478650
T. wenchuanense	HMAS 60239	JX267044	–	–

(Tiangen Biotech (Beijing) Co. Ltd.) 25 µl. PCR reactions were run as follows: an initial denaturation at 95 °C for 3 min, followed by 30 cycles at 95 °C for 2 min, 55 °C for 25 s, 72 °C for 2 min, and a final extension at 72 °C for 10 min. The PCR products were sent to Invitrogen Biotechnology Co. Ltd. (Beijing, China) for purifying, sequencing, and editing. The other sequence data of ITS rDNA included in this study were downloaded from GenBank. GenBank numbers are shown in Table 1.

Phylogenetic analyses

DNA sequences were aligned with Clustal X (Thompson et al. 1997). The alignment was manually adjusted with Se-Al v.2.03a (Rambaut 2000). The aligned dataset was analyzed with maximum parsimony (MP) using PAUP*4.0b10 (Swofford 2002). MP analysis was conducted using heuristic searches with 1000 replicates of random-addition sequence, tree bisection reconnection (TBR) branch swapping algorithm. All characters were equally weighted and unordered. Gaps were treated as missing data to minimize homology assumptions. A bootstrap (BP) analysis was performed with 1000 replicates, each with 10 random taxon addition sequences. TBR branch swapping was employed. The Bayesian analysis was performed with MrBayes 3.1.2 (Huelsenbeck et al. 2001; Ronquist & Huelsenbeck 2003) with two sets of four chains (one cold and three heated) and the stoprule option in effect, halting the analyses at an average standard deviation of split frequencies of 0.01. The sample frequency was set to 100, and the first 25% trees were removed as burn-in. Bayesian posterior probabilities (PP) were obtained from the 50% majority rule consensus of the remaining trees. *Tuber aestivum* was used as outgroup.

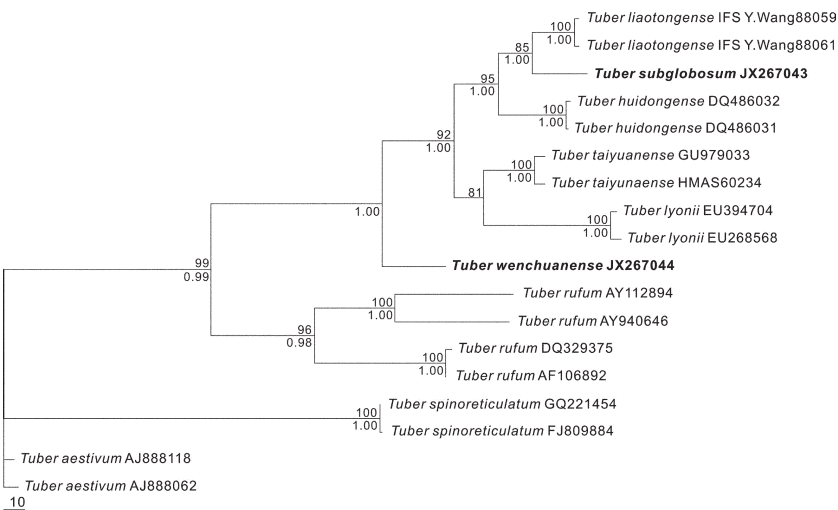

Fig.1. Phylogeny derived from maximum parsimony analysis of the ITS rDNA sequences of selected *Tuber* species with spino-reticulate ascospores, using *T. aestivum* as outgroup. Bootstrap values of more than 70% from 1000 replications are shown above the respective branches. Bayesian posterior probabilities were estimated and clades with PP>0.95 (95%) are marked under the branches.

Results

Molecular phylogenetics

The maximum parsimony analysis of ITS sequences produced one most parsimonious tree (Fig. 1) with a length (TL) = 708 steps, consistency index (CI) = 0.7345, retention index (RI) = 0.7968, homoplasy index (HI) = 0.2655, and rescaled consistency index (RC) = 0.5852.

The ITS sequence analysis (Fig. 1) revealed that the sequence of the new species *T. subglobosum* grouped in a clade with the *T. liaotongense* sequences with moderate support (BP = 85, PP = 1.00). The *T. wenchuanense* sequence was placed as a separate clade in *Tuber*.

Taxonomy

Tuber subglobosum L. Fan & C.L. Hou, **sp. nov.** Fig. 2

MycoBank MB 800576

Differs from other *Tuber* species by the combination of subglobose ascospores and spino-reticulate spore ornamentation.

Type: China. Sichuan Province, Huili County, in the soil under mixed woods, 20 Dec. 2010, Jin-Zhong Cao116 (**Holotype**, BJTC FAN153).

Etymology: *subglobosum* (Lat.), referring to the subglobose ascospores.

Ascomata subglobose, 1–2 cm in diam., firm, solid, surface very minutely flattened verrucose, glabrous, brown at maturity. Odor slight, not pungent. Peridium 200–300 μm thick, two layers; outer layer 100–150 μm, pseudoparenchymatous, composed of small subangular or subglobose cells mostly 7.5–15 μm in diam., with thickened and yellowish-brown walls, darker towards the outer surface; inner layer 100–150 μm, composed of intricately interwoven hyphae, hyaline, thin-walled, branched, septate, 2.5–5 μm in diam. Gleba grey brown to brown at maturity, marbled with numerous, narrow, branched, white to white-yellow veins continuous with inner peridium. Asci subglobose, ellipsoid, clavate, or irregular, hyaline, thick-walled, mostly 2–4-spored, rarely 1-spored, with a short or a very long stalk, 75–100 × 45–70 μm excluding stalk. Ascospores mostly subglobose, a few broadly ellipsoid, dark brown at maturity, 20–32.5 × 20–27.5 μm excluding ornamentation; ornamentation distinctly spino-reticulate, with spines 3–6 μm high, straight or bent at apex, the meshes generally 4–6 across the ascospore width.

Additional specimen examined: **China**. **Yunnan Province**, Huize County, in the soil under mixed woodland, 2 Nov. 2011, Shao-Ping Li 007 (BJTC FAN168).

Comments — *Tuber subglobosum* is very similar to *T. liaotongense*, an endemic species from Northern China. *Tuber liaotongense* also has subglobose to broad elliptic ascospores with spino-reticulate ornamentations, but its spines are much lower on the reticulum, making the ornamentation look like a typical reticulum (Cao et al. 2011). This alone is sufficient to distinguish the new

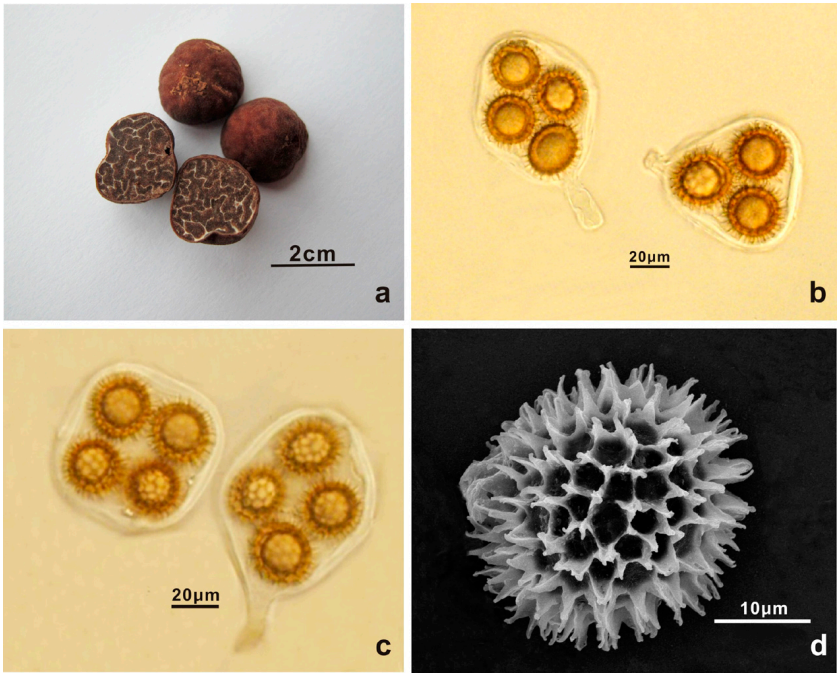

Fig.2. *Tuber subglobosum* (BJTC FAN153, holotype) a. Ascomata. b, c. Asci and ascospores observed under light microscope. d. Ascospore observed under SEM.

species. The phylogenetic analysis (Fig. 1) also grouped *T. subglobosum* in a clade with *T. liaotongense* but with only moderate support (BS = 85, PP = 1.00). This indicates that the two species are closely related but clearly distinct.

Other *Tuber* species with spino-reticulate ascospores differ from *T. subglobosum* by their more or less regular ellipsoid ascospores.

Tuber wenchuanense L. Fan & J.Z. Cao **sp. nov.** Fig.3

MycoBank MB 800577

Differs from the other *Tuber* species by its complete, deep and irregular spino-reticulate ascospore ornamentation with the spines connected to each other at both the bases and the tips with U-shaped ridges.

Type: China. Sichuan Province, Wenchuan County, in soil under *Larix mastersiana* Rehder & E.H. Wilson, 22 Sept. 1989, Bin-Cheng Zhang 618 (HMAS 60239, **Holotype**); Bin-Cheng Zhang 622 (**Isotype,** HMAS 60241A).

Etymology: *wenchuanense* (Lat.), referring to the type locality of the taxon.

Ascomata globose or subglobose, 0.5–1 cm in diam., grey brown when fresh, slightly pitted and whitish at the base, surface smooth. Odor not know

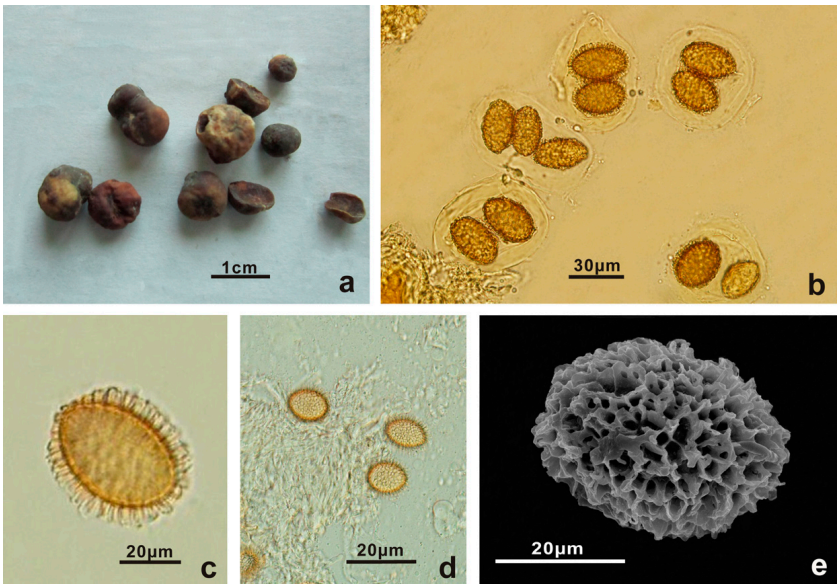

Fig.3. *Tuber wenchuanense* (HMAS 60239, holotype) a. Ascomata. b. Asci and ascospores observed under light microscope. c, d. Ascospore observed under light microscope. e. Ascospore observed under SEM.

when fresh. Peridium 200–250 μm thick, two layers; outer layer 50–100 μm, pseudoparenchymatous, composed of subglobose or subangular cells 5–15 μm in diam., with slightly thickened walls, yellowish-brown towards the surface; inner layer 150–200 μm, composed of intricately interwoven hyphae, hyaline, thin-walled, branched, septate, 2.5–5 μm diam. Gleba white, pale white or grey brown at maturity, marbled with numerous, narrow, branched, white veins continuous with inner peridium. Asci subglobose, ellipsoid or irregular, hyaline, thin-walled, 65–95 × 50–70 μm, sessile, 1–5-spored. Ascospores ellipsoid or broadly ellipsoid, yellow brown, covered with spines at first, then adjacent spines being joined by U-shaped ridges forming a complete irregular spino-reticulum, 25–45 × 17.5–30 μm excluding the ornamentation of 3–4 μm in height, occasionally on some ascospores the tips of spines remain isolated when the spines reaching up to 5–7.5 μm in height and reflexed at the tips, meshes numerous and various, typically 8–14 across the ascospore width.

Comments — The type specimen of *T. wenchuanense* in HMAS had been labeled as *T. lyonii*, a species originally from North America which, according to Trappe et al. (1996), is a species with ascospores with isolated spines or spines with bases partially connected by low ridges that form a complete or incomplete reticulum. However, our molecular analysis showed that the Chinese material

did not group in the same clade as *T. lyonii*, and instead belonged to a clade of its own (Fig. 1). Because of this and the unique ornamentation of its ascospores, we treat the Chinese material as a new species.

Acknowledgments

We are grateful to Prof. Wen-Ying Zhuang and Dr. Ian R. Hall for serving as the pre-submission reviewers. The study was supported by the NSFC (No. 30870008), the Beijing Natural Science Foundation (No. 5072006) and the Key Program of Beijing Education Commission (KZ201110028036).

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